The impact of emerging bioconjugation chemistries on radiopharmaceuticals

Fay, Rachael ; Holland, Jason P

Abstract: The use of radiolabeled antibodies, immunoglobulin fragments and other proteins are an increasingly important sector of research for diagnostic imaging and targeted radiotherapy in Nuclear Medicine. As with all radiopharmaceuticals, efficient radiochemistry is a prerequisite to clinical translation. For proteins, variations in the primary amino acid sequence, the secondary structures and tertiary folds, as well as differences in size, charge, polarity, lipophilicity, and the presence of post-translational modifications, add complexity to the system. The choice of radionuclide or chelate, and their impact on the thermodynamic, kinetic and metabolic stability of a radiotracer has attracted much attention but the chemistry by which the radionuclide is conjugated to the protein scaffold is of equal importance. Recently, a wealth of creative advances in protein ligation methods based on chemical, photochemical and enzyme-mediated processes have emerged. As radiochemists explore alternative bioconjugation strategies, this article considers their potential impact on radiotracer design.

DOI: https://doi.org/10.2967/jnumed.118.220806

Journal of Nuclear Medicine, published on March 22, 2019 as doi:10.2967/jnumed.118.220806

The impact of emerging bioconjugation chemistries on radiopharmaceuticals

Rachael Fay and Jason P. Holland*

University of Zurich, Department of Chemistry, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland

*** Corresponding Author:**

Prof. Dr Jason P. Holland

Tel: +41.44.63.53.990

ORCID: 0000-0002-0066-219X

E-mail: jason.holland@chem.uzh.ch

Website: www.hollandlab.org

First author:

Rachael Fay

E-mail: rachael.fay@chem.uzh.ch

Running Title: *Bioconjugation methods in radiosynthesis*

Words (Main text): 4048 incl. 48 references

ABSTRACT

The use of radiolabeled antibodies, immunoglobulin fragments and other proteins are an increasingly important sector of research for diagnostic imaging and targeted radiotherapy in Nuclear Medicine. As with all radiopharmaceuticals, efficient radiochemistry is a prerequisite to clinical translation. For proteins, variations in the primary amino acid sequence, the secondary structures and tertiary folds, as well as differences in size, charge, polarity, lipophilicity, and the presence of post-translational modifications, add complexity to the system. The choice of radionuclide or chelate, and their impact on the thermodynamic, kinetic and metabolic stability of a radiotracer has attracted much attention but the chemistry by which the radionuclide is conjugated to the protein scaffold is of equal importance. Recently, a wealth of creative advances in protein ligation methods based on chemical, photochemical and enzyme-mediated processes have emerged. As radiochemists explore alternative bioconjugation strategies, this article considers their potential impact on radiotracer design.

Keywords: Bioconjugation, immuno-PET, antibodies, protein ligation, enzymes, photoradiochemistry

INTRODUCTION

It has long been established that the precise nature of the chemical linker used to couple a small-molecule or peptide to a radiometal complex can have a profound impact on the properties of a radiotracer (*1*). Our recent work on coupling the ^{68}Ga-DFO-complex *via* two different linker strategies to the urea-based Glu-NH-C(O)-NH-Lys group for imaging prostate specific membrane antigen (PSMA) illustrates this point (*2*,*3*). When compared with the clinically translated agent, ^{68}Ga-PSMA-11, dramatic differences were observed in cellular binding, tumor targeting and whole-body distribution and excretion profiles of ^{68}Ga-DFO-Nsucc-PSMA and ^{68}Ga-DFO-pNCS-Bn-PSMA – radiotracers constructed using *N*-succinyl and thiourea-based chemistries, respectively. Specifically, the small and less lipophilic *N*-succinyl linker reduced PSMA binding affinity with a dissociation constant from cellular assays (K_d-value) of 26.4 ± 7.8 nM compared with a value of 13.6 ± 2.6 nM for the compound with the pNCS-Bn linker, and 2.89 ± 0.55 nM for ^{68}Ga-PSMA-11. These differences in PSMA binding affinity also led to pronounced differences the uptake, retention and excretion of these three radiotracers in mice bearing LNCaP tumors.

For radiotracers based on antibodies, and other proteins, less is known about the impact that linker methodology may have on the stability, pharmacokinetics and target specificity of the ensuing radiotracer. Until recently, our collective experience with radiolabeled antibodies has relied on a small handful of conjugation routes. Classic chemistries typically involve reactions of native cysteine or lysine residues (*4–11*). For example, thiolate groups of cysteine side chains readily undergo Michael addition reactions with maleimide groups, and the ε-NH_2 group of lysine side chains participate in nucleophilic reactions using activated esters or isothiocyanates. An overview of the essential structure of an antibody and various options for performing conjugation chemistry is shown in Fig. 1 (*4*,*12*). Note that highlighted functionalization sites (positions 1 to 6)

are not intended to be a comprehensive list of all chemically accessible features but rather give a flavor of the types of conjugation reactions that are currently being explored. The selection does not include the wide array of 'click' methods for which the reader is referred to the detailed review by Meyer *et al.*(*13*) The following sections discuss some of the features of recent chemical, photochemical and enzyme-mediated conjugation reactions with a particular emphasis on applications with antibodies (*14*,*15*). Some of the methods described are yet to be applied in radiosynthesis but illustrate the scope of reactions that harbor potential for future use.

CHEMICAL FUNCTIONALIZATION METHODS

In spite of the diverse range of new bioconjugation methods, it remains true that the vast majority of synthetic transformations rely on the native chemistry of cysteine (sulfhydryl) and lysine (primary amine) side-chains. Some of the reactions of these two amino acids are presented in Fig. 2.

In general, reactions at cysteines involve either derivatization of a single residue or sequential reduction of disulfides followed by substrate-induced re-bridging (Fig. 2A). In all cases, the chemistry features nucleophilic substitution or addition-elimination mechanisms using reagents bearing labile leaving groups. Conjugation with maleimide groups has been a mainstay of conjugation chemistry for many decades. Indeed, two United States Federal Drug Administration approved antibody-drug conjugates (ADCs) – brentuximab vedotin (Adcetris®, Seattle Genetics) and trastuzumab emtansine (Kadcyla®, Genentech / Roche) – employ this conjugation strategy. However, in cysteine-rich environments, the reaction is potentially reversible and experience has found that such conjugates are susceptible to chemical and metabolic instability *in vivo* leading to questions over long-term stability (*16*). Stability is of particular concern for

radiotracers based on antibodies which require extended circulation times for peak accumulation in tumor lesions and clearance from background organs. Notably, stability of maleimide-conjugated *in vivo* can be improved by ensuring that the 5-membered ring hydrolyses to the ring-opened succinic acid thioether – the rate of which can be increased by the use of electron-withdrawing substituents on the nitrogen atom (*16*). Related agents based on ^{18}F have also been introduced (*17*). Since two bonds are usually better than one (from a thermodynamic and kinetic perspective) disulfide re-bridging is also an attractive strategy to potentially overcome stability-related issues associated with cysteine functionalization (*18*). Re-bridging can be achieved with the reagents shown (Fig. 2A [right]) but variants of the photochemically induced thiol-ene and thiol-yne radical based coupling reactions are also potential options (*15*).

In many aspects, lysine-based conjugation reactions are more readily accessible than cysteine functionalization. A typical IgG_1 human antibody has around 90 lysines, many of which are solvent exposed and chemically accessible (*19*). In contrast, IgG_1 antibodies like trastuzumab have 16 cysteine residues, all of which are involved in inter- and intra-chain disulfide bridges. Only the four inter-chain disulfides are easily modified but require reduction before the thiolate residues become accessible for functionalization. Hence, lysine-based reactions are the most prevalent method used for chemical modification of antibodies (Fig. 2B). Radiolabeling antibodies at lysine residues takes one of two mechanistic forms: either *i*) direct conjugation of the radiolabel to the ε-NH_2 group, or *ii*) a 'tag-and-modify' approach in which the side chain is first tagged with another reactive group that facilitates bioorthogonal coupling to a tertiary reagent (*c.f.* numerous 'click'-based conjugation and pre-targeting studies) (*13*,*20–22*). From a clinical perspective, the high reproducibility and efficiency of lysine modification using activated esters or isothiocyanate-

based reagents make these the most prevalent methods used in the synthesis of clinical-grade radiolabelled antibodies (*23*).

An interesting variation on the 'tag-and-modify' route involves the use of radiolabeled sugars to functionalize proteins (*24–27*). For instance, in 2015, Rashidan *et al.* reported that ^{18}F-FDG could be substituted at the anomeric (C_1) position using tetrazine reagents bearing an RO-NH_2 group (Fig. 2C) (*25*). Reaction of the amine with the ring-opened aldehyde isomer of ^{18}F-FDG (catalyzed by the addition of *p*-phenylenediamine, pPDA) gave a stable oxime intermediate that was subsequently used in site-specific radiolabeling of an anti-Class II Major Histone Complex (MHC) antibody. This work also featured the use of site-specific enzyme-mediated tagging of the antibody using sortase-A (*vide infra*) to incorporate the transcyclooctene partner for strain-promoted inverse electron demand Diels Alder 'click' conjugation.

A related 'tag-and-modify' approach was reported by Vugts *et al.* who used traceless Staudinger ligation to label antibodies that were pre-modified with an azide group (*28*). A series of phosphine-based reagents were synthesized to allow conjugation of the chelates DFO and DOTA, or an ^{123}I-radiolabeled cubyl-phosphine to the mAb *via* the traceless approach. Use of the traceless Staudinger ligation was also explored in the context of antibody pre-targeting. Although derivatization of proteins using Staudinger ligation chemistry was successful, a number of drawbacks including slow biomolecular conjugation kinetics and metabolic instability of the phosphine groups *in vivo* will likely preclude further use of this chemistry for pre-targeting (*29*).

As an alternative conjugation method, our group recently reported the development of a photochemically-mediated conjugation and radiolabeling reaction based on chelates bearing aryl azide groups (Fig. 2D) (*30–32*). The process is distinct from almost all other conjugation chemistries in that the active reagent is formed *in situ* by irradiating the samples with ultra violet

light (~365 nm). Under irradiation, aryl azides spontaneously release N_2(g) and undergo rapid isomerization to give a ketenimine intermediate. The unique feature of this mechanism is that the ketenimine intermediate acts as a preferential electrophile for primary and secondary amines, thereby facilitating chemo-selective, lysine-based conjugation. Conceptually, photochemistry is an attractive foundation for bioconjugation reactions because, *i*) photoactivation can occur at wavelengths where most proteins do not absorb and also on a short time scale that minimizes potential damage to the underlying protein, *ii*) productive protein ligation occurs with high quantum efficiency and under ambient conditions so no heating is required, *iii*) the aryl azide reagents are cheap, easy to synthesize, and thermally stable up to ~60 ºC permitting long-term storage, and *iv*) photoradiochemistry permits the simultaneous, one-pot conjugation and radiolabeling of *non-purified* antibodies (i.e. antibodies in their standard GMP formulation) in <20 min. A combination of stability measurements, cellular assays, temporal PET imaging and biodistribution studies confirmed that the ^{89}Zr-DFO-azepin-trastuzumab obtained by photoradiochemistry was biochemically equivalent to ^{89}Zr-DFO-Nsucc-trastuzumab produced by a conventional thermochemical conjugation route (Fig. 3) (*32*,*33*). Recent work by Poot *et al*. introduced fully automated radiolabeling and purification of ^{89}Zr-radiolabelled antibodies, but as with manual synthesis, the approach still relies on the use of pre-conjugated protein (*34*). An additional advantage of our photoradiochemical method is that the conjugation step is potentially amenable to full automation which opens the possibility of using non-modified proteins as starting materials.

Native chemical ligation (NCL) was originally introduced in the 1990s by Kent and co-workers (Fig. 2E) (*14*,*35*,*36*). The NCL process is straight-forward and involves a reversible trans-esterification reaction initiated by nucleophilic attack of an *N*-terminal Cys-thiolate on an

engineered *C*-terminal thioester. The resulting activated thioester then undergoes a spontaneous and irreversible, intramolecular *S*-to-*N* acyl shift under physiological conditions to yield a peptide bond linkage. This approach is an elegant, chemo-specific and regio-specific method for facile derivatization of proteins but to the best of our knowledge, has yet to be applied in radiotracer design. The caveat is that *C*-terminal thioesters are required but recent efforts have improved access to these reagents (*14*,*37*).

ENZYME-MEDIATED FUNCTIONALIZATION METHODS

Beyond chemically-mediated transformations, several groups have begun to adapt the exquisite specificity and selectivity of enzymes for antibody (protein) functionalization and radiotracer synthesis. A wide array of enzyme-mediated reactions are available,(*14*) but three examples that have already been utilized in radiochemistry include applications of transglutaminase,(*4*,*38*) glycan modification(*8*,*39–42*) with multi-step transformations employing α-2,6-sialyltransferase, β-1,4-galactosidase and mutated β-1,4-galactosyltransferase, as well as sortase-A mediated conjugation using proteins engineered to express the LPXTG tags (Fig. 4) (*25*,*43*,*44*).

Conjugation reactions involving transglutaminase can occur at native Q295 glutamine residues in the C_H2 domain of IgG_1 antibodies (Fig. 4A). Alternatively, site-specific derivatization can be achieved at engineered protein tags (so-called ‘Q-tags’) which harbor a recognition sequence and an accessible glutamine residue that acts as a substrate for mutated transglutaminase enzymes (*45*).

Most antibodies are inherently glycoproteins (Fig. 1). Therefore, glycoengineering offers a fascinating approach for site-selective modification of antibodies. Different strategies for modifying glycans exist but the general features include, *i*) an enzymatic ‘cleaning step’ to

homogenize the presentation of glycans on the antibody sample, followed by *ii*) enzyme-mediated conjugation using a non-native substrate bearing either a bioorthogonal reactive group or a chelate for subsequent radiolabeling (Fig. 4B). Using this approach, Zeglis and co-workers reported a number of interesting applications ranging from pretargeted immuno-PET through to the synthesis of functionalized antibodies for multi-modal (PET and optical) imaging (*41*).

There is no doubt that the aforementioned examples of enzyme-mediated chemistry are elegant approaches to functionalize antibodies. However, for future clinical applications, it is crucial to ensure complete removal of the enzyme from the desired protein conjugate. Arguably one of the most exciting strategies involves the use of sortase-A transpeptidase (Fig. 4C). This approach is conceptually more challenging, because from the outset it involves the use of engineered proteins that display a specific LPXTG sequence. However, this tag guarantees site-selective functionalization. For example, Paterson *et al.* reported the use of sortase-A to couple a sarcophogine chelate (derivatized with a short polyethylene glycol linker and an *N*-terminal triglycine $(Gly)_3$ group) to an scFv fragment that targets the ligand-induced binding sites (LIBS) on the glycoprotein receptor GPIIb/IIIa (*43*). An additional feature of sortase-A conjugation is that the reactive components, including the sortase-A enzyme can be co-engineered to display a $(His)_6$-tag sequence which facilitates purification using standard affinity chromatography (*44*). If successful protein conjugation occurs, the $(His)_6$-tag can be designed to be automatically cleaved from the product (e.g. using augmented recognition *C*-terminal sequences such as LPXTG-$(His)_6$). At the same time, unreacted reagents retain their original $(His)_6$-tag and byproducts receive a $(His)_6$-tag *via* an exchange mechanism. Hence, purification of the desired protein from reagents and unwanted byproducts is a 'built-in' part of the sortase-A process.

Finally, it is worth noting that alternative conjugation strategies involving engineered fusion proteins exist (*46–48*). Here, the most familiar example involves fusion of streptavidin (53 kDa) to a protein of interest followed by site-specific conjugation with a substrate bearing a biotin-tag. However, other promising approaches include the use of SNAP-tag (~19 kDa), CLIP-tag (~19 kDa) and HaloTag (33 kDa) enzymes (Fig. 5). SNAP-tags and CLIP tags accept work *via* the same mechanism. Knight *et al.* used the HaloTag approach combined with an ^{111}In-radiolabeled complex to pretarget the non-internalizing TAG-72 biomarker expressed on LS174T colorectal cancer cells (*48*). Metal binding chelates featuring a fluorescence label and an alkylchloride group – which forms a covalent bond with the HaloTag enzyme – were developed. From a pretargeting perspective, HaloTags were found to be sufficiently reactive to allow specific accumulation in tumors. However, incorporation of long-chain aliphatic handles on the chelate component may potentially alter the lipophilicity and pharmacokinetic distribution of the reagents *in vivo*. In addition, fusion of larger proteins to an antibody has the potential to alter the distribution profile. Nevertheless, excellent tumor localization of the radioactivity was achieved in tumor-bearing mice. With further optimization, HaloTags and related fusion proteins will undoubtedly allow expansion of this technology to other substrates and targeting vectors.

CONCLUSIONS AND FUTURE DIRECTIONS

A wide range of alternative conjugation methods are making their way into the radiochemical sciences. Strategies involving bioorthogonal chemistry, photochemistry and enzyme-mediated processes all show promise for future radiotracer design. These new methods allow researchers to construct peptide- and protein-based radiotracers with unprecedented control over the chemo-selectivity and regio-selectivity of the conjugation step. Advances in site-specific conjugation

allow new immuno-PET tracers to be synthesized while minimizing the risk of compromising the distribution profile and immunoreactivity of the antibody or biomolecule by avoiding modification at undesirable sites. As new technologies are developed, it is worth keeping in mind that existing methods (namely, non-specific cysteine and lysine functionalization) are simple, work efficiently for standard antibodies, and are already part of routine clinical translation. Therefore, any alternative conjugation method should deliver advantages that standard non-specific chemistry cannot provide. Ideally, new methods should also be applicable to a diverse range of cancer targeting proteins. In this respect, selected chemistries described in this article have the potential to change the way which radiotracers are designed and produced, and may expand the range of immuno-PET for clinical imaging. It will be exciting to see how these technologies advance in the coming years.

ACKNOWLEDGEMENTS

JPH thanks the Swiss National Science Foundation (SNSF Professorship PP00P2_163683), the Swiss Cancer League (Krebsliga Schweiz; KLS-4257-08-2017), and the University of Zurich (UZH) for financial support. This project has received funding from the European Union's Horizon 2020 research and innovation programme / from the European Research Council under the Grant Agreement No 676904, ERC-StG-2015, NanoSCAN. We thank all members of the Radiochemistry and Imaging Science group at UZH for helpful discussions.

DISCLOSURE

No other potential conflict of interest relevant to this article was reported.

REFERENCES

1. Jamous M, Tamma ML, Gourni E, et al. PEG spacers of different length influence the biological profile of bombesin-based radiolabeled antagonists. *Nucl Med Biol*. 2014;41:464-470.

2. Gourni E, Canovas C, Goncalves V, Denat F, Meyer PT, Maecke HR. (R)-NODAGA-PSMA: A versatile precursor for radiometal labeling and nuclear imaging of PSMA-positive tumors. *PLoS One*. 2015;10:1-16.

3. Gourni E, Pozzo L Del, Bartholoma M, et al. Radiochemistry and preclinical PET Imaging of 68Ga-desferrioxamine radiotracers targeting prostate-specific membrane antigen. *Mol Imaging*. 2017;16:1-11.

4. Dennler P, Fischer E, Schibli R. Antibody conjugates: from heterogeneous populations to defined reagents. *Antibodies*. 2015;4:197-224.

5. McCombs JR, Owen SC. Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry. *AAPS J*. 2015;17:339-351.

6. Spicer CD, Davis BG. Selective chemical protein modification. *Nat Commun*. 2014;5(4740):1-14.

7. Chalker JM, Bernardes GJL, Davis BG. A "tag-and-modify" approach to site-selective protein modification. *Acc Chem Res*. 2011;44:730-741.

8. Adumeau P, Sharma SK, Brent C, Zeglis BM. Site-specifically labeled immunoconjugates for molecular imaging—Part 1: cysteine residues and glycans. *Mol Imaging Biol*. 2016;18:1-17.

9. Adumeau P, Sharma SK, Brent C, Zeglis BM. Site-specifically labeled immunoconjugates for molecular imaging—Part 2: peptide tags and unnatural amino acids. *Mol Imaging Biol*. 2016;18:153-165.

10. Boutureira O, Bernardes GJL. Advances in chemical protein modification. *Chem Rev*. 2015;115:2174-2195.

11. Krall N, Da Cruz FP, Boutureira O, Bernardes GJL. Site-selective protein-modification chemistry for basic biology and drug development. *Nat Chem*. 2016;8:103-113.

12. Morais M, Ma MT. Site-specific chelator-antibody conjugation for PET and SPECT imaging with radiometals. *Drug Discov Today Technol*. 2018:91-104.

13. Meyer JP, Adumeau P, Lewis JS, Zeglis BM. Click chemistry and radiochemistry: The first 10 years. *Bioconjug Chem*. 2016;27:2791-2807.

14. Baumann AL, Hackenberger CPR. Modern ligation methods to access natural and modified proteins. *Chimia (Aarau)*. 2018;72:802-808.

15. Xiao F, Zhang X, Lei X. Recent developments and applications of photoconjugation

chemistry. *Chim Int J Chem*. 2018;72:782-790.

16. Fontaine SD, Reid R, Robinson L, Ashley GW, Santi D V. Long-term stabilization of maleimide-thiol conjugates. *Bioconjug Chem*. 2015;26:145-152.

17. Chiotellis A, Sladojevich F, Mu L, et al. Novel chemoselective 18F-radiolabeling of thiol-containing biomolecules under mild aqueous conditions. *Chem Commun*. 2016;52:6083-6086.

18. Badescu G, Bryant P, Bird M, et al. Bridging disulfides for stable and defined antibody drug conjugates. *Bioconjug Chem*. 2014;25:1124-1136.

19. Jain N, Smith SW, Ghone S, Tomczuk B. Current ADC linker chemistry. *Pharm Res*. 2015;32:3526-3540.

20. Coursey BM, Collé R, Coursey JS. Standards of radium-226: From Marie Curie to the International Committee for Radionuclide Metrology. *Appl Radiat Isot*. 2002;56:5-13.

21. Billaud EMF, Belderbos S, Cleeren F, et al. Pretargeted PET imaging using a bioorthogonal 18F-Labeled trans-cyclooctene in an ovarian carcinoma model. *Bioconjug Chem*. 2017;28:2915-2920.

22. Denk C, Svatunek D, Filip T, et al. Development of a ^{18}F-labeled tetrazine with favorable pharmacokinetics for bioorthogonal PET imaging. *Angew Chemie - Int Ed*. 2014;53:9655-9659.

23. Vosjan MJWD, Perk LR, Visser GWM, et al. Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine. *Nat Protoc*. 2010;5:739-743.

24. Namavari M, Cheng Z, Zhang R, et al. A Novel Method for Direct Site-Specific Radiolabeling of Peptides Using [18F]FDG. *Bioconjug Chem*. 2009:432-436.

25. Rashidian M, Keliher EJ, Dougan M, et al. Use of 18F-2-fluorodeoxyglucose to label antibody fragments for immuno-positron emission tomography of pancreatic cancer. *ACS Cent Sci*. 2015;1:142-147.

26. Krishnan HS, Ma L, Vasdev N, Liang SH. 18F-Labeling of Sensitive Biomolecules for Positron Emission Tomography. *Chem - A Eur J*. 2017;23:15553-15577.

27. Elgland M, Nordeman P, Fyrner T, Antoni G, Nilsson KPR, Konradsson P. β-Configured clickable [^{18}F]FDGs as novel ^{18}F-fluoroglycosylation tools for PET. *New J Chem*. 2017;41:10231-10236.

28. Vugts DJ, Vervoort A, Stigter-Van Walsum M, et al. Synthesis of phosphine and antibody-azide probes for in vivo staudinger ligation in a pretargeted imaging and therapy approach. *Bioconjug Chem*. 2011;22:2072-2081.

29. Lang K, Chin JW. Bioorthogonal Reactions for Labeling Proteins. *ACS Chem Biol*. 2014;9:16-20.

30. Patra M, Eichenberger LS, Fischer G, Holland JP. Photochemical conjugation and one-pot radiolabelling of antibodies for immuno-PET. *Angew Chemie Int Ed*. 2019;58:1928-1933.

31. Eichenberger LS, Patra M, Holland JP. Photoactive chelates for radiolabelling proteins. *Chem Commun*. 2019;55:2257-2260.

32. Patra M, Klingler S, Eichenberger LS, Holland J. Simultaneous photoradiochemical labelling of antibodies for immuno-PET. *iScience*. 2019;in press. doi.org/10.1016/j.isci.2019.03.004

33. Holland JP, Caldas-Lopes E, Divilov V, et al. Measuring the pharmacodynamic effects of a novel Hsp90 inhibitor on HER2/neu expression in mice using 89Zr-DFO-trastuzumab. *PLoS One*. 2010;5:e8859.

34. Poot AJ, Adamzek KWA, Windhorst AD, et al. Fully automated zirconium-89 labeling and purification of antibodies. *J Nucl Med* . December 2018.

35. Dawson PE, Muir TW, Clark-Lewis I, Kent SBH. Synthesis of proteins by native chemical ligation. *Science (80-)*. 1994;266:776-779.

36. Conibear AC, Watson EE, Payne RJ, Becker CFW. Native chemical ligation in protein synthesis and semi-synthesis. *Chem Soc Rev*. 2018:9046-9068.

37. Burlina F, Morris C, Behrendt R, White P, Offer J. Simplifying native chemical ligation with an N-acylsulfonamide linker. *Chem Commun*. 2012;48:2579-2581.

38. Dennler P, Chiotellis A, Fischer E, et al. Transglutaminase-based chemo-enzymatic conjugation approach yields homogeneous antibody-drug conjugates. *Bioconjug Chem*. 2014;25:569-578.

39. Zhou Q, Stefano JE, Manning C, et al. Site-specific antibody-drug conjugation through glycoengineering. *Bioconjug Chem*. 2014;25:510-520.

40. Zeglis BM, Davis CB, Aggeler R, et al. Enzyme-mediated methodology for the site-specific radiolabeling of antibodies based on catalyst-free click chemistry. *Bioconjug Chem*. 2013;24:1057-1067.

41. Zeglis BM, Davis CB, Abdel-Atti D, et al. Chemoenzymatic strategy for the synthesis of site-specifically labeled immunoconjugates for multimodal PET and optical imaging. *Bioconjug Chem*. 2014;25:2123-2128.

42. Adumeau P, Vivier D, Sharma SK, et al. Site-Specifically Labeled Antibody-Drug Conjugate for Simultaneous Therapy and ImmunoPET. *Mol Pharm*. 2018;15:892-898.

43. Paterson BM, Alt K, Jeffery CM, et al. Enzyme-mediated site-specific bioconjugation of metal complexes to proteins: Sortase-mediated coupling of copper-64 to a single-chain antibody. *Angew Chemie - Int Ed*. 2014;53:6115-6119.

44. Massa S, Vikani N, Betti C, et al. Sortase A-mediated site-specific labeling of camelid single-domain antibody-fragments: a versatile strategy for multiple molecular imaging

modalities. *Contrast Media Mol Imaging*. 2016;11:328-339.

45. Rachel NM, Pelletier JN. Biotechnological applications of transglutaminases. *Biomolecules*. 2013;3:870-888.

46. Zhang Y, So MK, Loening AM, Yao H, Gambhir SS, Rao J. HaloTag protein-mediated site-specific conjugation of bioluminescent proteins to quantum dots. *Angew Chemie - Int Ed*. 2006;45:4936-4940.

47. England CG, Luo H, Cai W. HaloTag technology: a versatile platform for biomedical applications. *Bioconjug Chem*. 2015;26:975-986.

48. Knight JC, Mosley M, Uyeda HT, et al. In vivo pretargeted imaging of HER2 and TAG-72 expression using the HaloTag enzyme. *Mol Pharm*. 2017;14:2307-2313.

Figure 1. Structural features of a generic IgG_1 human antibody showing the various domains of the heavy and light chains, the antigen binding (CDR = complementary determining region), the Fab fragment, the crystallizable fragment (Fc), and the location of glycosylated residues. Reaction sites include (but are not limited to): (1) cysteine or reduced disulfides, (2) lysine functionalization, (3) *C*- or *N*-terminal chemistry, including the use of exogenous engineered sequences such as His_6, Myc-tag, HaloTag or SNAP-tags etc, (4) transglutaminase coupling, (5) enzyme-mediated glycan modification, and (6) bispecific antibodies with one arm selective for a metal ion complex and the other for the target of interest.

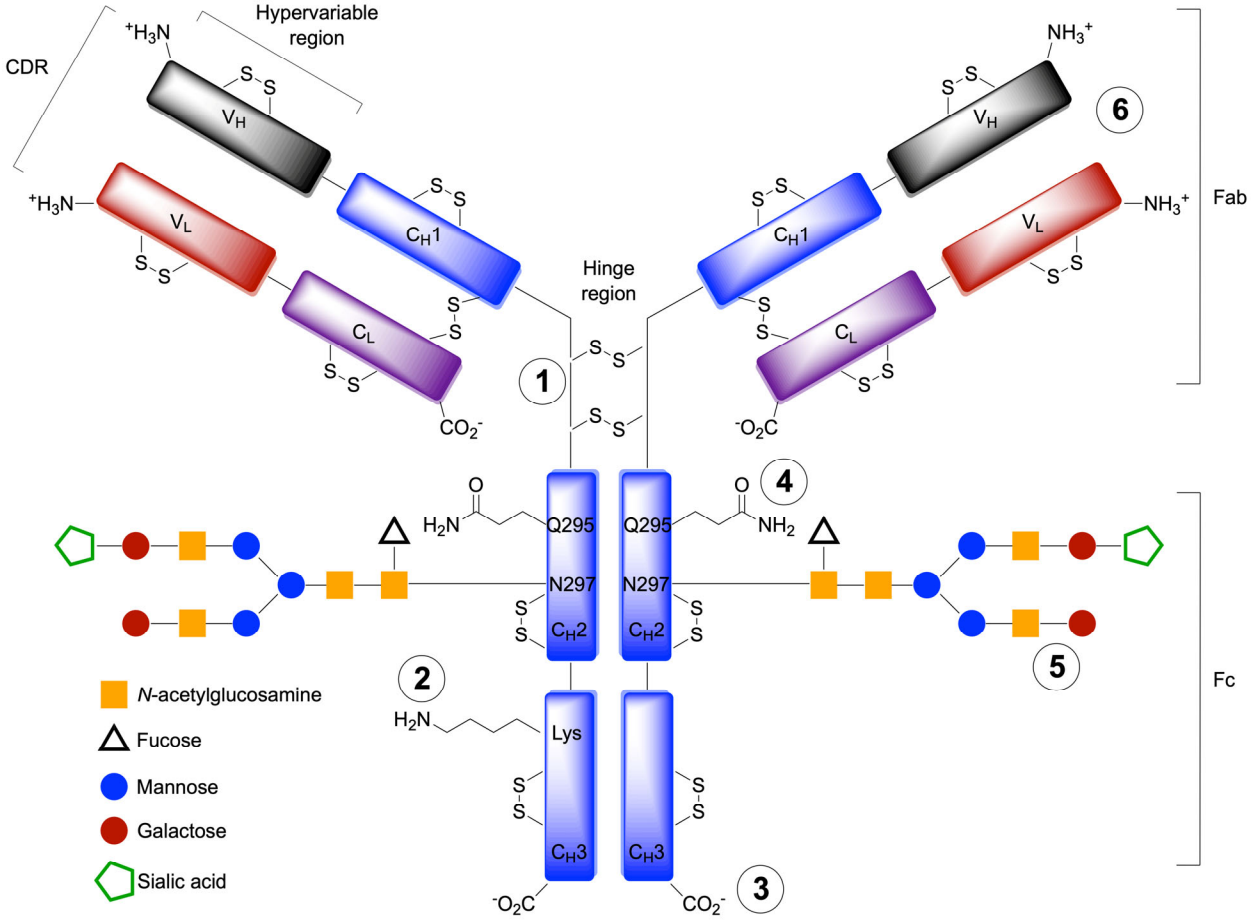


Figure 2. Illustration of some of the classic and modern chemical reactions for protein ligation. Note that the figure is not intended to be comprehensive but rather gives a flavor of the different types of reagents and transformations that are potentially useful in protein-based radiotracers synthesis.

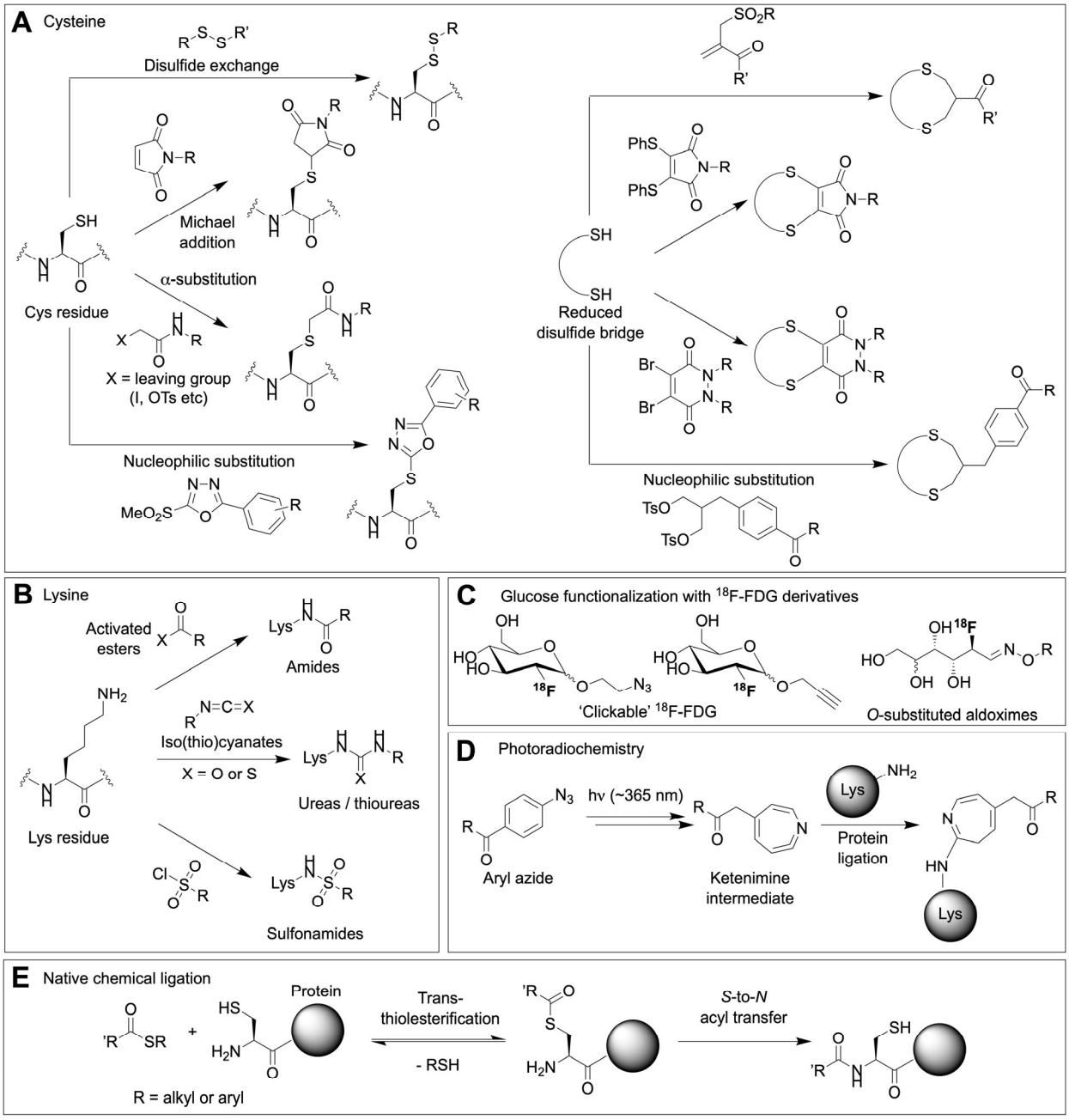


Figure 3. Comparison of PET images recorded in athymic nude mice that were administered with either (left) [^{89}Zr]ZrDFO-azepin-trastuzumab synthesized by a photochemical conjugation and radiolabeling process (unpublished data), and (right) [^{89}Zr]ZrDFO-Nsucc-trastuzumab synthesized *via* a conventional thermochemical route using *N*-succinyl (Nsucc) activated ester chemistry (*33*). No discernable differences were observed when comparing the PET images or the biodistribution data for the two radiotracers produced *via* different the photochemical or thermochemical routes.

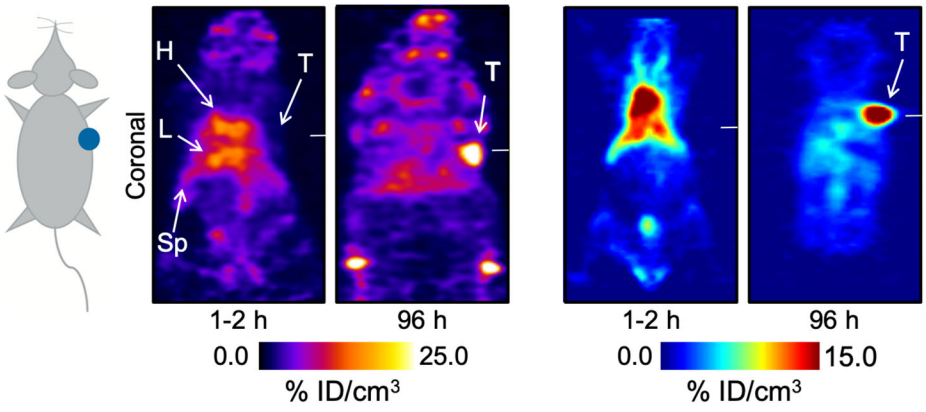


Figure 4. Representative examples of enzyme-mediated protein ligation reactions. (A) Transglutaminase reaction using primary amine substrates and either natural or engineered glutamine residues. (B) Multi-step glycan modification. (C) Site-specific sortase-A mediated conjugation using the LPXTG tag (where X = any amino acid and the coupled substrate contains an *N*-terminal poly-glycine handle.

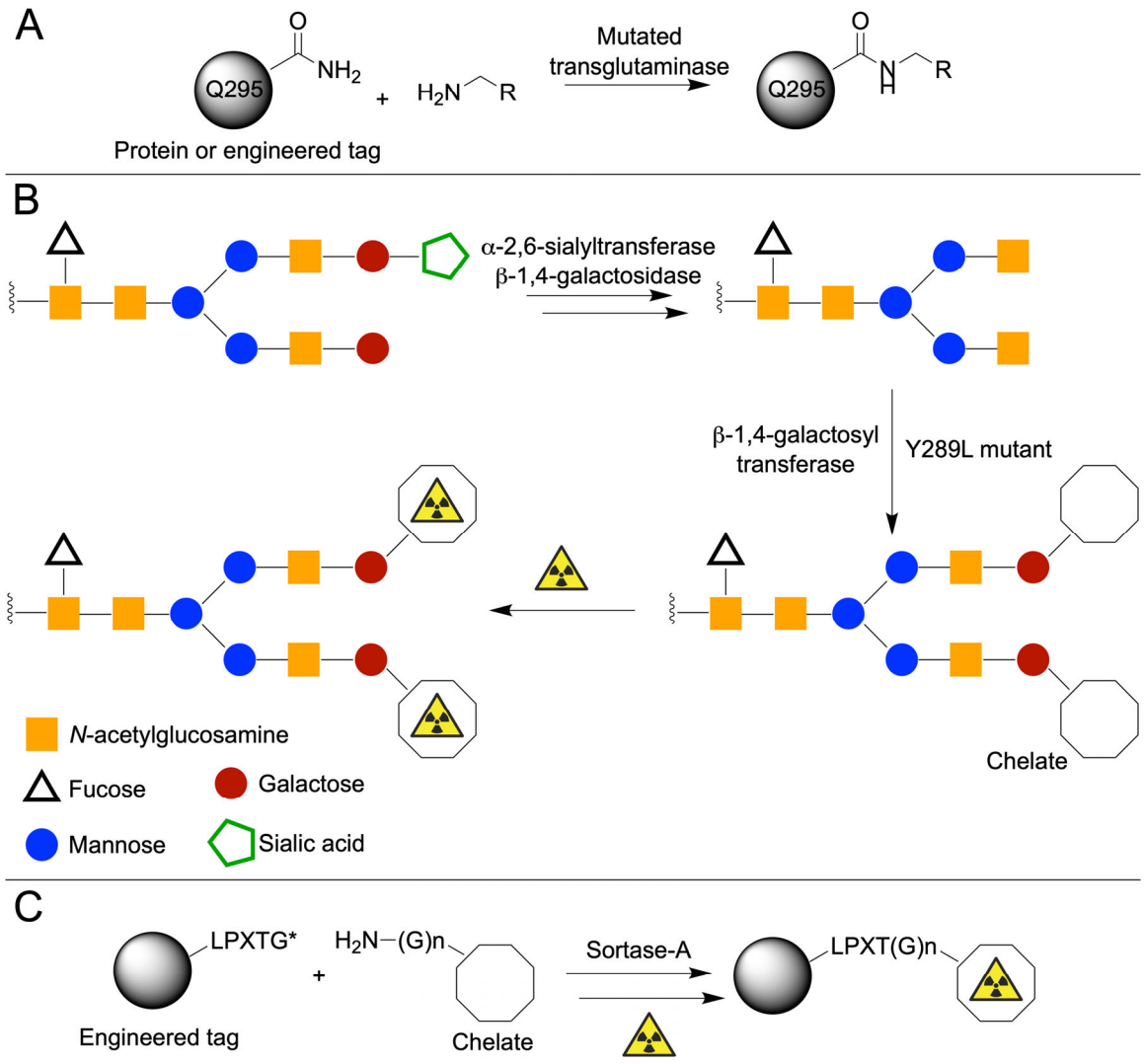
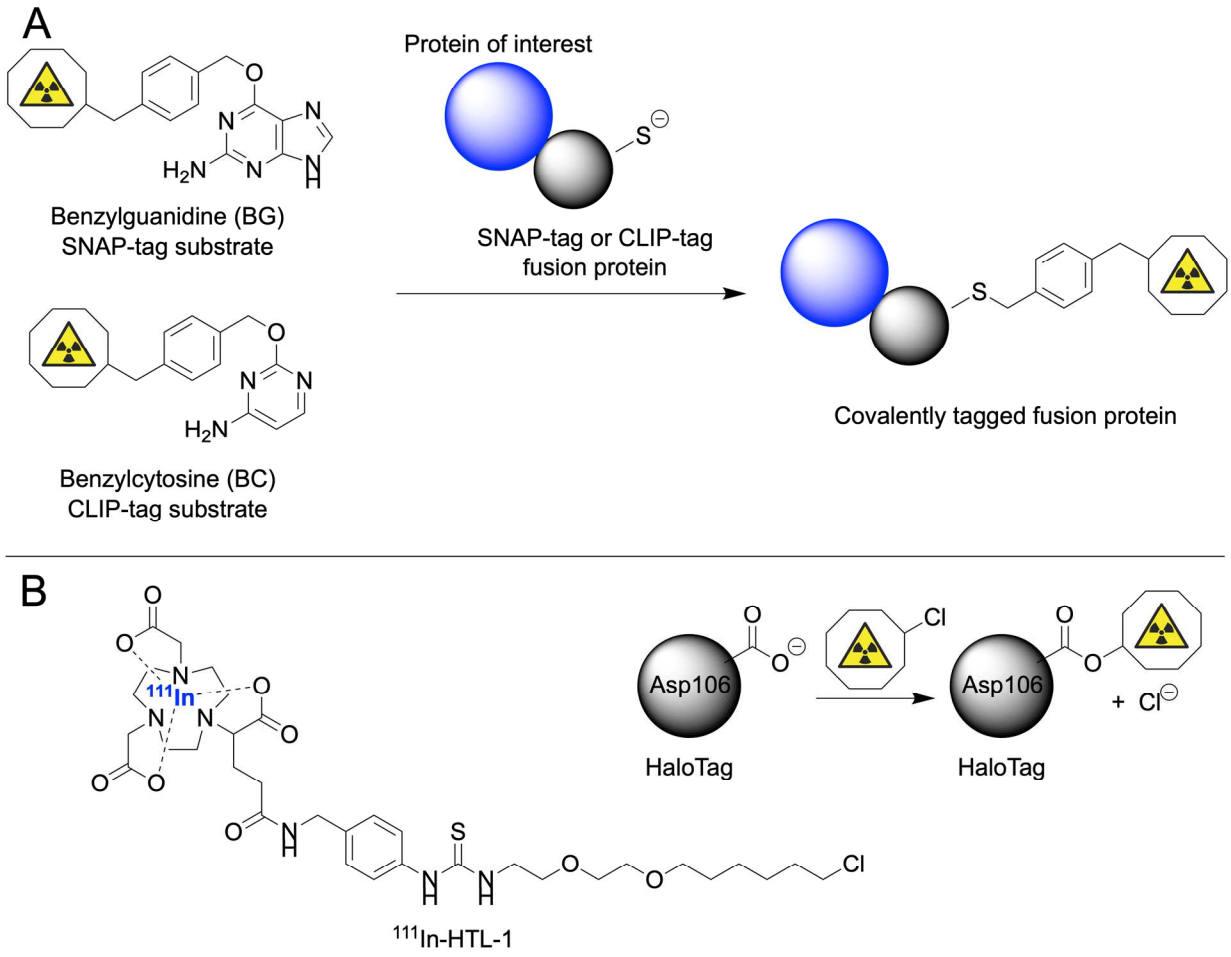


Figure 5. Illustration of the substrates and covalent bioconjugation reactions on fusion proteins involving, (A) the use of SNAP-tags or CLIP-tags with benzylguanosyl (BG) and benzylcytosyl (BC) substrates, respectively, and (B) covalent bond formation between the Asp106 residue in the mutated active site of a HaloTag and an ^{111}In-radiolabeled alkylchloride substrate (^{111}In-HTL-1) (*48*).



The impact of emerging bioconjugation chemistries on radiopharmaceuticals

Rachael Fay and Jason P. Holland

J Nucl Med.
Published online: March 22, 2019.
Doi: 10.2967/jnumed.118.220806

The Journal of Nuclear Medicine is published monthly.
SNMMI | Society of Nuclear Medicine and Molecular Imaging
1850 Samuel Morse Drive, Reston, VA 20190.
(Print ISSN: 0161-5505, Online ISSN: 2159-662X)